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ORIGINAL ARTICLE

Dietary L-proline supplementation confers immunostimulatory effects on inactivated *Pasteurella multocida* vaccine immunized mice

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Abstract This study was conducted to determine the immunostimulatory effect of L-proline on inactivated vaccine immunized mice. Ninety-five female KM mice were randomly divided into five groups: (1) mice received dietary supplementation with 0.4 % L-proline and immunized with inactivated vaccine (V–P group); (2) mice received dietary supplementation with 0.3 % L-alanine (isonitrogenous control) and immunized with inactivated vaccine (V–A group, negative control); (3) mice were immunized with inactivated vaccine with oil adjuvant (V–O group, positive control); (4) mice were immunized with inactivated vaccine with aluminum hydroxide adjuvant (V–H group, positive control); (5) mice immunized with phosphate-buffered saline (control group). All mice were dead in the control group between 36 and 48 h post infection. Mice in the V–P group showed 100 % protection after challenge with *P. multocida* serotype A (CQ2) at dose of 4.4×10^5 CFU (2LD50). Meanwhile, serum antibody titers in the V–P group were higher than those in the V–A group before infection and those in the V–A and V–O groups at 36 h post infection. Moreover, serum IL-1β levels in the V–P group were lower than those in V–O group. Furthermore, serum GSH-PX levels in the V–P group were higher than those in the V–A and V–O groups. Collectively, dietary proline supplementation confers beneficial immunostimulatory effects in inactivated *P. multocida* vaccine immunized mice.

Keywords Proline · *Pasteurella multocida* · Immunostimulatory effect · Adjuvant

Abbreviations

BSA	Bovine serum albumin
GSH-PX	Glutathione peroxidase
IL	Interleukin
PBS	Phosphate-buffered saline
TNF-α	Tumor necrosis factor alpha

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Introduction

Any substance that helps the vaccine to stimulate the immune system's response to a target antigen, but does not in itself confer immunity, is referred to as an immunologic adjuvant. Formulation of an effective vaccine, especially for purified, synthetic and subunit vaccine, generally requires an appropriate adjuvant which should be stable, biodegradable, cheap to produce, immunologically inert, have a long shelf life, and promote both the humoral and cellular immune response to an antigen (Sun et al. 2008). However, minimizing toxicity remains as one of the major challenges in adjuvant research and use. For example, aluminium hydroxide, which is common adjuvant sold in the word and has been used in vaccine for over 70 years, is poor adjuvant in inducing cellular immune response and exerts many side effects, such as IgE production, allergenicity and neurotoxicity (Clapp et al. 2011; Goto et al. 1993). Thus, there is a need for identification of new adjuvants that are both safe and efficacious for the use to produce a variety of vaccines.

Traditionally, proline is categorized as a nutritionally nonessential amino acids in mammals (Wu 2009). However, similar to arginine, glutamine and alpha-ketoglutarate, growing evidence shows that proline is a nutritionally essential amino acid for poultry, young mammals, fish and wounded subjects (Li et al. 2007, 2009; Liu et al. 2012; Tan et al. 2009, 2011; Ren et al. 2013; Wu et al. 2011; Yao et al. 2008, 2012). Proline is also a key regulator of multiple biochemical and physiological processes in cells. For example, proline is a signaling molecule, a sensor of cellular energy status, and a source of pyrroline-5-carboxylate and superoxide anion (a free radical) participating in redox reactions in humans and animals (Phang et al. 2008, 2010). In addition, proline plays an important role in conceptus metabolism, growth and development, as well as a potential treatment for intrauterine growth restriction, which is a significant problem in both human medicine and animal production (Rezaei et al. 2011; Wu et al. 2008). Furthermore, proline plays a role in protecting lymphocytes from apoptosis, stimulating cell growth and promoting antibody production (Duval et al. 1991) and a proline-rich polypeptide complex may affect not only adaptive immunity, but also innate immunity, thereby regulating secretions of inflammatory mediators of Zablocka et al. (2010). Collectively, proline exerts an important role in physiological and immunological function. However, not much is known about immunostimulatory effects of proline on vaccine-immunized mammals. Thus, this study was conducted to evaluate immunostimulatory effects of dietary L-proline supplementation in mice immunized with the inactivated *Pasteurella multocida* vaccine.

Materials and methods

Preparation of the bacterium and inactivated vaccine

The *P. multocida* serotype A (CQ2) strain used in the present study was isolated from the lung tissue of clinically infected cattle, which was dead with pneumonia. The bacterium was regarded as *P. multocida* serotype with 16 s rRNA sequence and multiplex capsular PCR typing system (Townsend et al. 2001). The bacteria isolate was cultured in the Martin's broth agar medium containing 5 % horse serum. The pathogenicity of the isolate was tested in healthy mice by intraperitoneal inoculation the 2 days culture, and all the mice were succumbed to infection between 36 and 48 h post-infection. Bacteria re-isolated from these infected mice were used to prepare the inactivated vaccine. The bacterial inoculum was identified to be *P. multocida* serotype A using the PCR method and biochemical characteristics. Before animal experiments, the *P. multocida* serotype A-inactivated whole cell vaccine was prepared. In brief, bacterial colonies isolated from the liver of mice were cultured in the Martin's agar at 37 °C overnight, and then an isolate colony from the overnight static culture was transferred to 100 ml of the Martin's broth to incubate at 37 °C for 12 h with 85 rpm. Then, *P. multocida* serotype A strains were inactivated by addition of 0.4 % formalin to the culture mix (10^9 CFU/ml) and incubation at 37 °C for 24 h with continuous agitation.

Experimental design

Ninety-five female KM mice (body weight 18–22 g) were obtained from Laboratory Animal Center of Third Military Medical University, Chongqing, China. The mice were housed in a pathogen-free mouse colony (temperature, 20–30 °C; relative humidity, 45–60 %; lighting cycle, 12 h/day) and had free access to food and drinking water. Animals were randomly divided into five groups ($n = 19$ per group): (1) mice received dietary supplementation with 0.4 % L-proline (Ajinomoto Inc., Tokyo, Japan) (basal diet + 0.4 % L-proline) from day 0 and immunized with inactivated vaccines at dose of 10^9 CFU at day 15 and 20 (V–P group); (2) mice received dietary supplementation with 0.3 % L-alanine (isonitrogenous control) from day 0 and immunized with inactivated vaccines at dose of 10^9 CFU at days 15 and 20 (V–A group, negative control); (3) mice were immunized with inactivated vaccines with oil adjuvant at dose of 10^9 CFU at days 15 and 20 (V–O group, positive control); (4) mice were immunized with inactivated vaccines with aluminum hydroxide adjuvant at dose of 10^9 CFU at days 15 and 20 (V–H group, positive control); (5) mice immunized with the same volume of phosphate-buffered saline at days 15 and 20 (control group). As

previous study, the amino acid content in the basal diet was measured using Automatic Amino Acid Analyzer (Yin et al. 2011). This basal diet contained 17 % protein. At day 34, all of the mice were challenged by an intraperitoneal injection of *P. multocida* serotype A (CQ2) at the dose of 4.4×10^5 CFU (2LD50). Ten mice in each group were used to calculate survival rate, whereas the others were euthanized to collect serum at 36 h post-infection for determination of cytokine levels, glutathione peroxidase (GSH-PX) activity, and antibody titers. Serum antibody titers in all groups were also analyzed at day 34 before challenge. This study was performed according to the guidelines of the Laboratory Animal Ethical Commission of the Chinese Academy of Sciences.

Analysis of serum cytokine levels

Interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) in serum were measured using ELISA kits in accordance with the manufacturer's instructions (CUSABIO BIOTECH CO., Ltd., China). An aliquot (100 µl) of the sample or standard was added to duplicate wells of the microtiter plate, which had been pre-coated with antibody (Yin et al. 2008). The buffer was used as a negative control. The plate was incubated for 2 h at 37 °C. A 100 µl of biotin antibody was added to each well after the removal of the liquid and incubated for 1 h at 37 °C. The wells were washed three times with 200 µl of the washing buffer. A 100 µl quantity of HRP–avidin was then added to each well for 1 h at 37 °C. After the final wash, an aliquot (90 µl) of the TMB substrate was added and incubated for 30 min in the dark at 37 °C. The reaction was stopped with 50 µl of the terminating solution and absorbance measured at 450 nm.

Serum GSH-PX activity

Serum GSH-PX activity was measured using spectrophotometric kits in accordance with the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China).

Analysis of antibodies

Enzyme-linked immunosorbent assays were used for the detection of antibodies. Before detection, antigens were prepared. In brief, 10 ml bacteria culture was crushed by ultrasonic wave, and then diluted with bicarbonate buffer (pH 7.4). A 96-well plate was coated by antigens (100 µl of the prepared antigens) and incubated at 4 °C for 16 h for adsorption. Primary antisera were diluted 1:40 in PBS containing 0.5 % Tween-20 and 1 % bovine serum albumin (BSA). Following five washings, horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, USA) diluted 1:10000 in PBS-T containing 1 % BSA was added to each well and the plates were incubated at 37 °C for 3 h. After the final wash, an aliquot (100 µl) of the TMB substrate was added and incubated for 15 min in the dark at 37 °C. The reaction was stopped with 50 µl of the terminating solution and absorbance measured at 450 nm.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software. Data are expressed as mean values ± standard error of the mean. Multiple comparisons were performed using the Student–Newman–Keuls method (Ren et al. 2013). Differences were considered significant at $P < 0.05$ (Wei et al. 2012).

Results

Cytokine profile and GSH-PX activity

Mouse serum IL-1 β, IL-6 and IL-8 levels were measured at 36 h after infection. As indicated in Table 1, immunization with inactivated vaccines with oil adjuvant increased ($P < 0.01$) serum IL-1β levels, in comparison to dietary proline or alanine supplementation or the control group. Serum IL-1β levels in the V–H group were ($P < 0.01$) than those in the control group; while no difference was found among the other groups. Unlike IL-1β, serum IL-6 and IL-8 levels in the control group were higher ($P < 0.01$) than those in other groups. Serum IL-8 levels in the V–A group were higher ($P < 0.05$) than those in the V–P, V–O and V–H groups; no difference was detected among these groups (Table 1). Furthermore, serum GSH-PX levels in the V–P, V–H and control groups were higher ($P < 0.01$) than those in the V–A and V–O groups; while no difference was found among other groups (Fig. 1).

Serum antibody titers

Serum antibody titers against *P. multocida* serotype A were measured before and after challenged with *P. multocida* serotype A. As indicated in Table 2, serum antibody titers in the V–P, V–A,V–O and V–H groups were much higher ($P < 0.01$) than those in the control group. Immunization with inactivated vaccines and dietary proline supplementation increased ($P < 0.05$) serum antibody titers, when compared with the immunization with inactivated vaccines and dietary alanine supplementation before infection; furthermore, serum antibody titers in the V–P and V–H groups were higher ($P < 0.05$) than those in the V–A and V–O

Table 1 Serum cytokine levels in different groups after a challenge with *P. multocida* serotype A (CQ2) at dose of 4.4 × 105 CFU

Group	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-1β (pg/ml)
V–P	4.26 ± 1.26^{B}	210.00 ± 30.93^{C}	392.03 ± 39.24^{BC}
V–A	2.39 ± 1.59^{B}	337.88 ± 55.86^{B}	393.43 ± 16.57^{BC}
V–O	2.13 ± 1.96^{B}	176.53 ± 15.91^{C}	568.50 ± 32.29^{A}
V–H	10.83 ± 4.08^{B}	176.38 ± 28.48^{C}	$497.07 \pm 51.07^{A,B}$
Control	2955.6 ± 200.11^{A}	6631.93 ± 91.90^{A}	327.61 ± 31.72^{C}

Five groups were: (1) mice received dietary supplementation with 0.4 % L-proline and immunized with inactivated vaccine (V–P group); (2) mice received dietary supplementation with 0.3 % L-alanine (isonitrogenous control) and immunized with inactivated vaccine (V–A group, negative control); (3) mice were immunized with inactivated vaccine with oil adjuvant (V–O group, positive control); (4) mice were immunized with inactivated vaccine with aluminum hydroxide adjuvant (V–H group, positive control); (5) mice immunized with phosphate-buffered saline (control group). Data are mean ± SEM, $n = 6$. Mean values sharing different superscripts within each cytokine differ ($P < 0.001$)

IL-1β Interleukin-1 beta, *IL-6* Interleukin-6, *IL-8* Interleukin-8

Table 2 Serum antibody titers against *P. multocida* serotype A in different groups (OD450)

Group	BI	AI
V–P	0.33 ± 0.09^{a}	0.36 ± 0.008^{a}
V–A	0.29 ± 0.01^{b}	0.31 ± 0.03^{b}
V–O	0.30 ± 0.007^{ab}	0.30 ± 0.003^{b}
V–H	0.32 ± 0.01^{ab}	0.36 ± 0.01^{a}
Control	0.10 ± 0.004*	0.14 ± 0.03*

Five groups were: (1) mice received dietary supplementation with 0.4 % L-proline and immunized with inactivated vaccine (V–P group); (2) mice received dietary supplementation with 0.3 % L-alanine (isonitrogenous control) and immunized with inactivated vaccine (V–A group, negative control); (3) mice were immunized with inactivated vaccine with oil adjuvant (V–O group, positive control); (4) mice were immunized with inactivated vaccine with aluminum hydroxide adjuvant (V–H group, positive control); (5) mice immunized with phosphate-buffered saline (control group). Data are mean ± SEM, $n = 6$. Mean values sharing different superscripts within columns differ ($P < 0.05$)

BI before infected with *P. multocida* serotype A, *AI* 36 h post infected with *P. multocida* serotype A

* $P < 0.01$ versus other groups in each columns

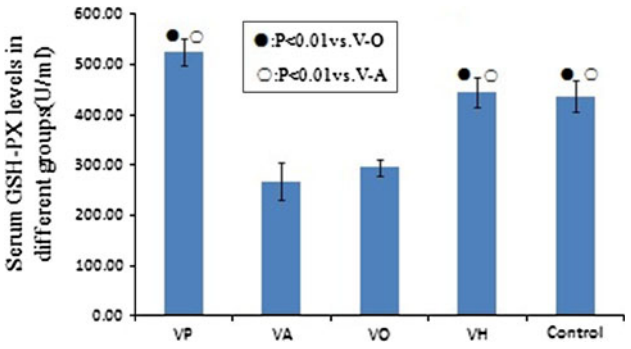

Fig. 1 Serum GSH-PX levels in different groups after challenge with *P. multocida* serotype A (CQ2) at dose of 4.4×10^5 CFU (U/ml). Five groups were: (1) mice received dietary supplementation with 0.4 % L-proline and immunized with inactivated vaccine (V–P group); (2) mice received dietary supplementation with 0.3 % L-alanine (isonitrogenous control) and immunized with inactivated vaccine (V–A group, negative control); (3) mice were immunized with inactivated vaccine with oil adjuvant (V–O group, positive control); (4) mice were immunized with inactivated vaccine with aluminum hydroxide adjuvant (V–H group, positive control); (5) mice immunized with phosphate-buffered saline (control group). GSH-PX: glutathione peroxidase. Data are mean ± SEM, $n = 6$

groups at 36 h post infection; while no difference was detected among the other groups.

Survival rates

The survival rates of immunized mice were calculated every day after infection with *P. multocida* serotype A. All mice were dead in the control group between 36 and 48 h post infection, whereas two mice died in each of the V–A and V–O adjuvant groups and no death was observed in the V–P or V–H group (Fig. 2).

Discussion

There is growing interest in the dietary requirements of traditionally classified "nutritionally nonessential amino acids" by animals (Hou et al. 2012; Li et al. 2011; Tan et al. 2012a, b; Wu 2010a, b, 2013). This study was conducted to evaluate the immunostimulatory effects of dietary L-proline supplementation on inactivated *P. multocida* vaccine-immunized mice. Based on the challenge experiment, no death was observed in the V–P and V–H groups, and only 10.5 % of mice died in the V–A or the V–O group, in striking contract to 100 % mortality in the control group. These compelling results indicate that proline, similar to the Al $(OH)_3$ adjuvant, exerts a significant immunostimulatory effect on inactivated *P. multocida* vaccine-immunized mice. To illustrate this promising result, we analyzed serum antibody titers before and post the challenge to study the stimulatory effects of proline in the systemic humoral immune response. A significant increase was observed in all the mice immunized with the inactivated *P. multocida* vaccine, in comparison with the mice immunized with PBS. Notably, the most promising results are that antibody titers in the V–P group were much higher than those in the V–A group on day 34 and that antibody titers in the V–P group were also higher than those in the V–O and V–A groups at 36 h post challenge. Similarly, serum antibody titers in the V–H group were

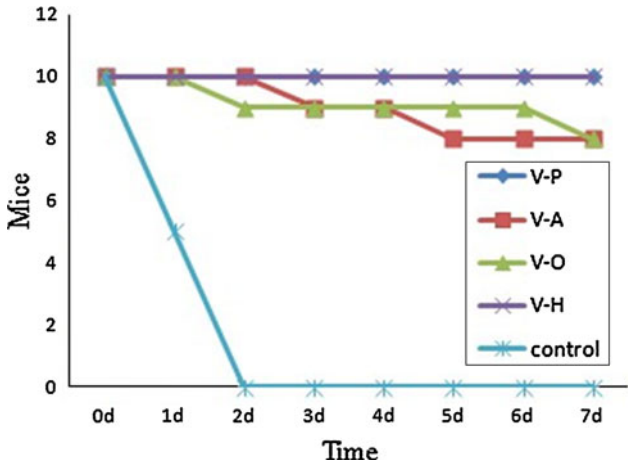

Fig. 2 The mortality in each group after challenge with *P. multocida* serotype A (CQ2) at dose of 4.4×10^5 CFU. Mice were immunized with inactivated vaccines and dietary 0.4 % proline supplementation as V–P group; mice were immunized with inactivated vaccines and dietary 0.3 % alanine supplementation as V–A group; mice were immunized with inactivated vaccines and oil adjuvant as V–O group; mice were immunized with inactivated vaccines and aluminum hydroxide adjuvant as V–H group; mice immunized with PBS as control group ($n = 10$/group)

higher than those in the V–O and V–A groups at 36 h post challenge. These results indicate that dietary supplementation with 0.4 % proline increases serum antibody titers in the inactivated *P. multocida* vaccine-immunized mice. The findings also help explain the observation that the mortality rate in the control group was 100 %, while no mortality was observed in the V–P or V–H group. At present, the molecular mechanisms responsible for the effects of proline on enhancing antibody production are unknown.

The production of cytokines is closely related to immune responses and mortality in animals (Praveena et al. 2010; Ren et al. 2012). A novel and important finding of this study is that dietary supplementation with proline affected concentrations of inflammatory cytokines in serum. Particularly, IL-1β is one of the pivotal early response pro-inflammatory cytokines that, through up- or down regulation of other cytokine, enables organisms to respond to infectious non-self challenges and induces a cascade of effects leading to inflammation (Dinarello 1997). IL-6 plays a very complex role in biological events, including immune responses, hematopoiesis, and regulation of the endocrine and nervous systems (Biffl et al. 1996; Naugler and Karin 2008). IL-8 is often associated with inflammation by acting preferentially on neutrophils (Baggiolini et al. 1995). Similarly, we found that serum IL-6 and IL-8 levels in the control group were much higher than those in other groups and that serum IL-8 levels in the V–A group is higher than those in the V–P, V–O and V–H groups. These compelling results are in consistent with the previous studies that indicated that serum concentrations of pro-inflammatory cytokines increased in mice after a challenge with *P. multocida* serotype A, while high antibody titers in other groups protected mice from high levels of pro-inflammatory cytokines (Praveena et al. 2010; Ren et al. 2012). Moreover, serum IL-1β levels in the V–P and V–H groups were neither the lowest nor the highest. This intermediate levels of IL-1β may contribute to the high protection against mortality in the V–P and V–H groups because an optimal concentration of IL-1β functions as a mediator of the host inflammatory response to infection and other inflammatory stimuli, whereas larger quantities of IL-1β induce many negative effects, including inducing synthesis of acute-phase plasma proteins, initiating metabolic wasting (Dinarello 1997). Although serum IL-1β levels in the V–A group was similar to those in the V–P group, only 10.5 % of mice in the V–A group died, indicating that factors, including low antibody titers, may also affect the protection.

As an indicator of anti-oxidative ability, serum GSH-PX levels in each group were determined to explain the clinical results. GSH-PX is an important peroxidase widely spread in the body (Wang et al. 2009). It can remove harmful peroxide metabolites from cells and block the lipid peroxidation chain reaction, thereby protecting the integrity of cell membrane structure and function (Wu et al. 2004). Consistent with the clinical results, serum GSH-PX levels in the V–P and V–H groups were substantially higher than those in the V–A and V–O groups. As alluded to above concerning IL-1β, a mere increase in serum GSH-PX levels in the control group could not ensure high protection. A major reason for high GSH-PX levels in the proline group may be that proline can scavenge free radicals, and thus has vital antioxidant properties (Kaul et al. 2008). In fact, there are reports that proline concentration increases markedly in response to cellular oxidative stress (Verbruggen and Hermans 2008).

Collectively, our results clearly demonstrate that dietary proline supplementation provides an immunostimulatory effect in inactivated *P. multocida* vaccine-immunized mice. The underlying mechanism may involve a crucial role for proline in modulating multiple biochemical, physiological and immunological processes in humans and animals by acting as a signaling molecule, a sensor of cellular energy status, and a source of pyrroline-5-carboxylate and superoxide anion (Phang and Liu 2012; Wang et al. 2012; Wu et al. 2011). In addition to proline itself, other substances produced from proline metabolism, such as arginine, glutamine and H_2O_2, may also play a vital role in immune function (Dai et al. 2012a, b; Gao et al. 2012; Hou et al. 2011; Li et al. 2007; Xi et al. 2012). Arginine, de novo synthesis from proline, is essential substance for T-lymphocyte responses for low L-arginine levels down-regulated the CD3 ξ chain (Bronte et al. 2003; Rodriguez

et al. 2003; Munder et al. 2006; Peranzoni et al. 2007). Glutamine is another metabolite that is crucial for optimal nutrition and immune response in animals (Li et al. 2007), including mice (Ren et al. 2011). Moreover, H_2O_2, a major product of proline oxidation, is a signaling molecule and a cytotoxic agent toward pathogenic bacteria (Rezaei et al. 2013a, b; Shi et al. 2004). Further studies are warranted to fully understand the molecular mechanisms for the adjuvant effect of proline in mammals.

In conclusion, dietary proline supplementation, like the traditionally used aluminum hydroxide adjuvant, increases serum antibody titers, ameliorates the serum cytokines profile, and elevates serum GSH-PX levels in inactivated *P. multocida* vaccine-immunized mice. These beneficial results eventually contribute to the full protection after a challenge with *P. multocida* serotype A. To our knowledge, this is the first study to determine the adjuvant effect of proline in mammals. Our findings have important implications for preventive medicine in humans and animals.

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Conflict of interest The authors declare that they have no conflict of interest.

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